PB90-117219

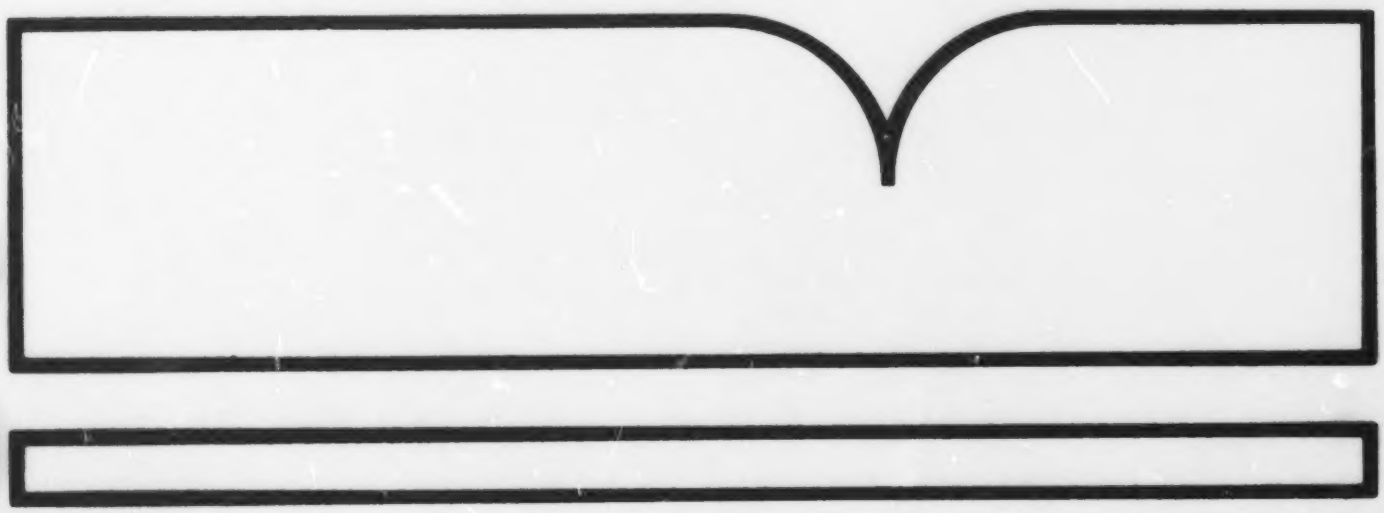
Sequential Reductive Dehalogenation of Chloroanilines by Microorganisms from a Methanogenic Aquifer

Oklahoma Univ., Norman

Prepared for:

Robert S. Kerr Environmental Research Lab., Ada, OK

1989



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TECHNICAL REPORT DATA

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1. REPORT NO.	2.	3.
EPA/600/J-89/105		PB90-117219

4. TITLE AND SUBTITLE

SEQUENTIAL REDUCTIVE DEHALOGENATION OF CHLOROANILINES BY MICROORGANISMS FROM A METHANOGENIC AQUIFER

5. REPORT DATE

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS

The University of Oklahoma, Dept. of Botany & Microbiology
770 Van Vleet Oval
Norman, OK 73019

10. PROGRAM ELEMENT NO.

ABPC1A

11. CONTRACT/GRANT NO.

CR-812808

12. SPONSORING AGENCY NAME AND ADDRESS

Robert S. Kerr Environmental Research Lab. - Ada, OK
U.S. Environmental Protection Agency
P.O. Box 1198
Ada, OK 74820

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE

EPA/600/15

15. SUPPLEMENTARY NOTES

Published: ENVIRONMENTAL SCIENCE & TECHNOLOGY, Vol. 23, No. 7, pp 848-852
July 1989.

16. ABSTRACT

the authors

Chloroaniline-based compounds are widely used chemicals and important contaminants of aquatic and terrestrial environments. We have found that chloroanilines can be biologically dehalogenated in polluted aquifers when methanogenic, but not sulfate-reducing conditions prevail. The halogens are replaced by protons in a series of reductive steps catalyzed by microorganisms. The sequential release of halogens from the para and ortho position of 2,3,4,5-tetrachloroaniline (2,3,4,5-tetraCA) resulted in the formation of 2,3,5-trichloroaniline (2,3,5-triCA) and eventually 3,5-dichloroaniline (3,5-diCA). Similarly, when 3,4-diCA was used as a parent substrate, it was transformed to 3-chloroaniline (3-CA). Metabolites and end products were identified by their chromatgraphic mobility and their mass spectral fragmentation pattern. This reaction helps suggest novel bioremediation approaches for aquifers and other environments contaminated with these chemicals.

con't on pg 848

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
U.S. DEPARTMENT OF COMMERCE
NATIONAL TECHNICAL INFORMATION SERVICE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	7
	20. SECURITY CLASS (This page)	22. PRICE
	UNCLASSIFIED	

Environ. Sci. Technol. 1989, 23, 848–852

EPA/600/J-89/103

Sequential Reductive Dehalogenation of Chloroanilines by Microorganisms from a Methanogenic Aquifer

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■ Chloroaniline-based compounds are widely used chemicals and important contaminants of aquatic and terrestrial environments. We have found that chloroanilines can be biologically dehalogenated in polluted aquifers when methanogenic, but not sulfate-reducing conditions prevail. The halogens are replaced by protons in a series of reductive steps catalyzed by microorganisms. The sequential release of halogens from the para and ortho position of 2,3,4,5-tetrachloroaniline (2,3,4,5-tetraCA) resulted in the formation of 2,3,5-trichloroaniline (2,3,5-triCA) and eventually 3,5-dichloroaniline (3,5-diCA). Similarly, when 3,4-diCA was used as a parent substrate, it was transformed to 3-chloroaniline (3-CA). Metabolites and end products were identified by their chromatographic mobility and their mass spectral fragmentation pattern. This reaction helps suggest novel bioremediation approaches for aquifers and other environments contaminated with these chemicals.

Introduction

Halogenated pesticides and other organic chemicals are pollutants of increasing concern to society (*1–5*) because they are found in major environmental compartments including rainwater (*6*), surface water (*7*), soil (*8*), and ground water (*9, 10*). Because of their known persistence (*11–17*) and toxicity characteristics (*18, 19*), the chloroanilines are a class of contaminants of major environmental concern. The breakdown of phenylurea, acylanilide, and phenylcarbamate pesticides contributes to the burden of chloroanilines in soil and the terrestrial subsurface (*11–17*). In addition, the chemical or biological reduction of azo dyes and halogenated nitroaromatic herbicides, explosives, and nitropyrenes can result in the formation of chloroanilines (*20–24*). Furthermore, chloroanilines are themselves an important class of industrial chemicals used in the manufacture of pesticides, plastics, pharmaceuticals, and dyes (*8, 25*). General surveys of the occurrence of organic compounds in ground water have identified the chloroanilines as important pollutants (*4, 5*).

The aerobic catabolism of chloroanilines by microorganisms is reasonably well characterized (*26–33*), but in the absence of molecular oxygen, the fate of these contaminants is virtually unknown (*34*). Particular concern has focused on the metabolic fate of these chemicals in aquifers, since this resource represents the major potable water supply for about half of the U.S. population. We therefore studied the metabolism of chloroanilines in samples taken from a shallow anoxic aquifer. We found that these substrates were reductively dehalogenated and we could relate these findings to the prevailing ecological conditions. This is the first report describing the metabolic fate of haloanilines under anaerobic conditions.

Experimental Section

Sampling and Incubation of Aquifer Slurries. Aquifer material and site water were sampled as previously described from two sites adjacent to the Norman Municipal Landfill (*35*). The samples were placed in an anaerobic glovebox where 50 g of aquifer material and 50 mL of site water were aseptically transferred to sterile glass serum bottles. The bottles were closed with butyl rubber stoppers, and the reducing conditions were controlled by addition of 1 mM sodium sulfide. The initial headspace of the bottles was a mixture of N_2/CO_2 (80%/20%). Resazurin was added as a redox indicator. Anaerobic and filter-sterilized (0.2 μm) stock solutions of chloroanilines (1 mM) were prepared in distilled water with the exception of the more hydrophobic triCA and tetraCA. Methanol stock solutions were made for 2,3,4-triCA (2 M) and 2,3,4,5-tetraCA (100 mM). All substrates were aseptically added to aquifer slurries to result in an initial concentration of ~50 μM. Experiments were performed in duplicate, and autoclaved aquifer slurries (121 °C, 15 lb/in.^{2}, 180 min) served as controls. The bottles were incubated upside-down in the dark at room temperature. The biodegradation of each substrate was evaluated individually. Unless otherwise noted, the loss of a substrate or its conversion to a metabolite was observed in both replicates. Each replicate was analyzed separately and substrate or metabolite concentrations represent mean values.

HPLC Analysis. The analysis of the substrates and their products was conducted by HPLC (reversed phase) and detected by UV absorption at 280 or 300 nm (solvent metering pump, Model 110 A, Beckman; UV–detector Model 165, Beckman, C18 column, 0.4 × 25 cm, Alltech). Acetonitrile/acetate buffer (50 mM, pH 4.5) was chosen as a mobile phase at ratios of 9/6 or 8/7. The former solvent ratio was chosen for the analysis of tri- and tetrachloroaniline and associated metabolites and retention times are given in minutes: 7.68 (2,3,4,5-tetraCA), 5.44 (2,3,4-triCA), 5.84 (2,4,5-triCA), 5.17 (3,4,5-triCA), 3.92 (3,4-diCA), 4.46 (3,5-diCA), 4.52 (2,5-diCA), 4.49 (2,4-diCA), 4.24 (2,3-diCA), 4.74 (2,6-diCA). The analysis of mono- and dichloroanilines employed the other solvent buffer ratio and retention times were 4.91 (3,4-diCA), 4.12 (2-CA), 3.90 (3-CA), and 3.77 (4-CA) min.

External standards were employed for determination of chemical concentrations. The concentration of 2,3,5-triCA was calculated from a 2,4,5-triCA standard since the former substrate was not commercially available and the latter exhibited a similar chromatographic mobility and UV absorption characteristics. The experimental standard deviation, including sampling and analysis, was less than 10%.

Mass Spectrometry. Samples (1 mL) were taken from the aquifer slurries and centrifuged (3 min, 8000*g*). The supernatant (pH ~7.0) was extracted with 1 mL of diethyl ether, and the organic phase was dried over Na_2SO_4 after it was transferred to a 2-mL glass vial. The extracts were concentrated under a nitrogen gas stream to a final volume of about 50–100 μL and analyzed with a Hewlett-Packard gas chromatograph (Model 5890) equipped with a mass selective detector (Model 5970) and a DB-5 fused silica capillary column (film thickness, 1 μm; inner diameter, 0.32 mm; length, 60 m). The GC/MS was operated with the computer system 59970 MS ChemStation (Hewlett-Packard). Approximately 3 μL of the ether extracts was injected (splitless mode, 30 s) at an injector temperature of 250 °C and a column temperature of 40 °C. The column

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attached at these positions. Chlorophenols possess analogous resonance structures, and the preferential removal of ortho and para halogens from these substrates has been observed (*9*, *46*). In a consistent fashion, when diuron and several other 3,4-dichloro-substituted aromatic pesticides were incubated under anaerobic conditions, they were microbiologically transformed by the reductive removal of para-substituted halogens (*47*, *48*).

However, anaerobic microorganisms can also remove meta-substituted aryl halides from various substrates. For instance, pentachlorophenol as well as lesser halogenated phenols could be reductively dehalogenated at all positions relative to the hydroxy group (for review, see ref 9). Furthermore, the dehalogenation of the Agent Orange pesticide, 2,4,5-T (2,4,5-trichlorophenoxyacetate), has been demonstrated to occur at either the meta or the para position (*38*, *39*). In this respect it is interesting to note that when methanogenic aquifer slurries were repeatedly spiked with 2,3,4,5-tetraCA, two primary dehalogenation reactions were observed. The first was as described above and involved the removal of a para halogen to produce 2,3,5-triCA. However, an additional metabolite was detected by HPLC and tentatively identified as the 2,4,5-triCA (data not shown). This isomer could only have been produced by a meta dehalogenation reaction.

An independent study of J. Struijs and J. Rogers (personal communication) showed that a series of dichloroanilines were biotransformed by reductive dehalogenation in anoxic pond sediment. Interestingly, preexposure of the sediment to chlorophenols resulted in the immediate transformation of the added dichloroanilines without a lag or adaptation period. Consistent with our study, the resulting monochloroanilines were not metabolized further under these conditions.

Since methanol was used as a carrier in some experiments, its role as a potential secondary substrate for the dehalogenating microorganisms must be considered. However, the biotransformation of 3,4-diCA was observed even without the methanol carrier. This finding shows that methanol is not essential for the dehalogenation process. Conceivably, methanol or other forms of organic matter normally present in the ground water could act as suitable electron donors to support aryl dehalogenation reactions or to accelerate the rate of metabolism.

Our results may also help clarify the fate of the pesticide pentachloronitrobenzene in anaerobic environments. Two studies have demonstrated the relatively fast reduction of this substrate to pentachloroaniline in flooded soil or in soil exposed to a nitrogen atmosphere (*49*, *50*). Recently, Aschbacher and Feil (*51*) observed traces of penta- and tetrachloroaniline in the urine of ruminants treated with a single oral dose of pentachloronitrobenzene. These findings might be explained by an initial nitro group reduction reaction followed by a reductive dehalogenation reaction of pentachloroaniline in a manner similar to 2,3,4,5-tetraCA and 3,4-diCA.

Conclusions

This study demonstrates the reductive dehalogenation of chloroanilines in aquifer slurries from a methanogenic but not from a sulfate-reducing site. The accumulation of the dehalogenated products occurred only in anaerobic nonsterile incubation mixtures, indicating the biological nature of the process.

This study also suggests a strategy for the bioremediation of ground waters, soils, wastewaters, or other environments contaminated with chloroanilines. Conceivably, a treatment process designed to initially maintain methanogenic conditions could be followed by an aerobic incubation phase. During the first step, aniline or lesser halogenated anilines would presumably accumulate. The complete oxidation of the latter compounds would occur during the second stage where oxygen would serve both as a coreactant and as an electron acceptor for the catalyzing microorganisms (*26*–*33*). Such an approach has been previously used to demonstrate the mineralization of pesticides like methoxychlor (*52*) and will likely prove successful with diverse substrates including tetrachloroethylene (*53*). Such strategies represent a potential biotechnological approach to the cleanup of contaminated areas in which the environment is engineered rather than the requisite microorganisms.

Acknowledgments

We thank T. Townsend and R. Beeman for the technical assisstance and the helpful discussions. We also thank M. J. McInerny, P. J. Reynolds, and K. Nicholas for reviewing the manuscript.

Registry No. 2-Chloroaniline, 95-51-2; 3-chloroaniline, 108-42-9; 4-chloroaniline, 106-47-8; 3,4-dichloroaniline, 95-76-1; 2,3,4-trichloroaniline, 634-67-3; 2,3,4,5-tetrachloroaniline, 634-83-3; 2,3,5-trichloroaniline, 18487-39-3; 3,5-dichloroaniline, 626-43-7.

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Table I. Estimated First-Order Decay Coefficients (k_1; month^{-1}) for the Anaerobic Biotransformation of Chlorinated Anilines Incubated for 8 Months in Methanogenic (M) or Sulfate-Reducing (SR) Aquifer Slurries

aniline compd	biotransformation rate (k_1)	
	SR incubtns	M incubtns
2-CA	<0.01	<0.01
3-CA	<0.01	<0.01
4-CA	<0.01	<0.01
3,4-diCA	<0.01	0.34
2,3,4-triCA	<0.01	<0.01[a] (2.9)
2,3,4,5-tetraCA	<0.01	1.36
2,3,5-triCA[b]	ND[c]	0.50
3,5-diCA[b]	ND[c]	<0.10

[a] Concentrations in sterile and nonsterile slurries were not significantly different after 8 months of incubation, but 2,3,4-triCA was completely transformed within 10 months. The rate given in parentheses reflects the later phase of degradation. [b] Metabolite of 2,3,4,5-tetrachloroaniline. [c] ND, not determined.

temperature was increased at a rate of 5 °C/min to 250 °C and held for 10 min. Helium served as the carrier gas at a flow rate of 60 mL/min (0.8 bar) and a split of 1:60. The interface column temperature was adjusted to 150 °C. The electron impact detector was operated at 70 eV and the scanning rate was ~2/s. The metabolites were identified by comparison of their spectra with authentic standards or by the National Bureau of Standards library spectra. The library (Data Base Revision 1.0, 1986) was supplied by Hewlett-Packard with the gas chromatograph/mass selective detector.

Results

Adaptation and Rates of Chloroaniline Degradation. The biotransformation of chloroanilines was studied with samples from two sites within a shallow unconfined aquifer polluted by leachate from a municipal landfill (*35*, *36*). Both sites have been well characterized and designated as either methanogenic (M) or sulfate-reducing (SR) environments according to their sulfate concentration, methane production rate, and bacteriological enumeration and activity assays (*35*). Aquifer slurries of sediment and site ground water were incubated in glass serum bottles with a variety of chloroanilines (Table I). The bottles were initially placed under anaerobic conditions, and reducing conditions were ensured and monitored with the amendment of sulfide and resazurin, respectively. Subsamples (3 mL) were taken from replicate serum bottles for HPLC analysis immediately following the chloroaniline amendment and after 1, 3, and 8 months. Autoclaved aquifer slurries served as controls.

Following an 8-month incubation period, biotransformation of chloroanilines was detected in aquifer slurries from the M site, but no corresponding transformation occurred in SR site slurries (Table I). A lag or adaptation period (*37*) of at least 3 months and 1 month was observed for 3,4-diCA and 2,3,4,5-tetraCA, respectively. After the lag period, the two chloroanilines disappeared with estimated first-order decay coefficients of 0.34 and 1.36 month^{-1}. 2,3,4-TriCA was also completely biotransformed after a somewhat longer incubation period with an estimated first-order decay coefficient of the same magnitude (Table I). These rate constants are conservative since they are corrected for the abiotic loss of parent substrate measured in the sterile controls. Within 8 months, 50% of the 3,4-diCA, and ~60% of the 2,3,4,5-tetraCA remained undetected in sterile controls. This is not surprising since polyhalogenated anilines show higher abiotic losses than lesser halogenated anilines, probably due to their higher hydrophobicity (high octanol/water coefficient), and therefore stronger sorption to aquifer solids and possibly the bottle closures. However, calculation of the product formation rates (see below) resulted in values comparable to the disappearance rates given in Table I. In contrast to the di-, tri-, and tetraCA, the three monochloroaniline isomers were not significantly metabolized (<10% of the initial concentration) within the course of the experiment (Table I). A second addition of parent substrate (3,4-diCA and 2,3,4,5-tetraCA) to actively degrading aquifer slurries resulted in complete chloroaniline degradation within 1 month, presumably resulting from the enrichment of an active microbial population.

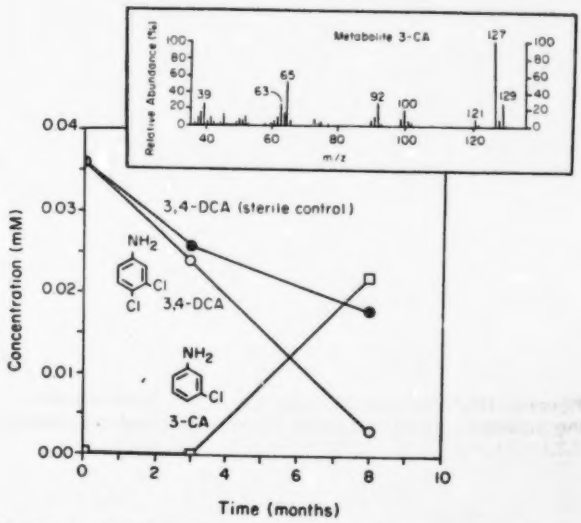

Figure 1. Dehalogenation of 3,4-diCA to 3-CA by anaerobic microorganisms in methanogenic aquifer slurries. The inset shows the spectrum of the metabolite obtained upon subsequent GC/MS analysis.

Biotransformation of 3,4-DiCA. Concomitant with the disappearance of 3,4-diCA in nonsterile aquifer slurries was the formation of a metabolite that coeluted during HPLC analysis with 3-CA but not with 2-CA or 4-CA (Figure 1). The metabolite was subjected to capillary GC/MS analysis and found to exhibit a clear one-chlorine isotopic abundance pattern at m/z 127 (Figure 1 inset). Other major mass spectral features include peaks at m/z 100 (HCN), 99 (HCN/H), 92 (Cl), and 65 (HCN/Cl). The only structure consistent with these mass spectral features and HPLC analysis is 3-CA. A mass spectral comparison of the metabolite with authentic 3-CA and a library spectrum of the same compound confirmed its identity as the reductively dehalogenated product of 3,4-diCA. No metabolites were detected in anaerobic autoclaved aquifer slurries. Between the third and eighth month of incubation, 24 μM 3,4-diCA was transformed and 22 μM 3-CA accumulated, accounting for 92% of the parent substrate loss (Figure 1). The residual 8% 3,4-diCA was presumably abiotically lost from the incubation vessels since the sterile controls also showed some disappearance of 3,4-diCA during the course of incubation.

Biotransformation of 2,3,4,5-TetraCA. When 2,3,4,5-tetraCA served as the parent substrate, two metabolites could be detected with time (Figure 2). The tetrachloroaniline (RT = 7.7 min) disappeared within 3 months from the aquifer slurries, and a more polar compound (metabolite A, RT = 6.2 min) transiently accumulated. The intermediate eventually disappeared and another even more polar compound was detected by HPLC

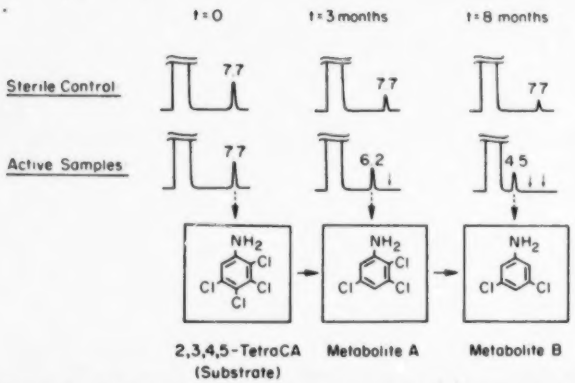

Figure 2. HPLC chromatograms illustrating the biotransformation of the substrate 2,3,4,5-tetraCA to the dehalogenated metabolites 2,3,5-triCA and 3,5-diCA in methanogenic aquifer slurries.

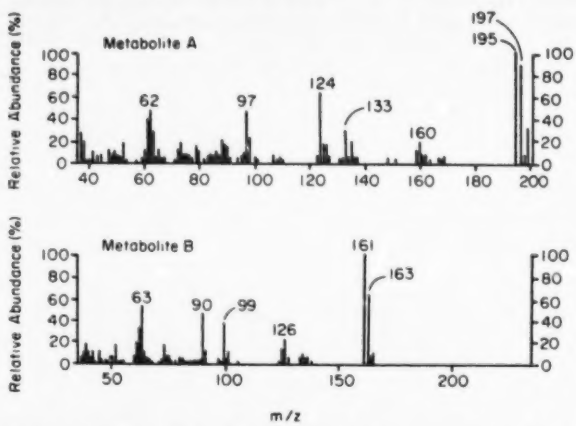

Figure 3. Mass spectral profiles of metabolites A and B formed during the anaerobic biotransformation of 2,3,4,5-tetraCA in methanogenic aquifer slurries.

(metabolite B, RT = 4.5 min). Our chromatographic analysis showed that metabolite A possessed a retention time different from the commercially available trichloroanilines (2,3,4-triCA, 2,4,5-triCA, 3,4,5-triCA). However, gas chromatographic/mass spectrometric analysis of metabolite A conclusively identified it as a trichloroaniline (Figure 3A). The mass spectrum contains a clear three-chlorine isotopic abundance pattern at m/z 195, a two-chlorine pattern at m/z 160 and 133, and a single chlorine pattern at m/z 124. The fragmentation pattern contains major abundances at m/z 160 (Cl), 133 (HCN/Cl), and 124 (2Cl/H). Considering the mass spectral and HPLC analysis, we conclude metabolite A to be 2,3,5-triCA. This isomer was then biotransformed to metabolite B.

The HPLC analysis of metabolite B revealed that this compound coeluted with 3,5-diCA (see Experimental Section; Figure 2), but not with 2,5-diCA. However, the elution profile of 2,4-diCA was similar to that of 3,5-diCA. Further analysis of the compounds by capillary GC separated 2,4-diCA (RT = 31.10 min) and 3,5-diCA (RT = 33.15 min) and revealed that metabolite B cochromatographed with 3,5-diCA. Mass spectral analysis of metabolite B conclusively identified it as dichloroaniline (Figure 3B). The mass spectrum exhibited a typical two-chlorine isotopic abundance pattern at m/z 161. The fragmentation of atoms or atom groups in the mass spectrometer resulted in the major peaks at m/z 126 (Cl), 125 (Cl/H), 99 (HCN/Cl), and 90 (2Cl/H). The metabolite B spectrum quality was greater than a 98% match with a library spectrum of 3,5-diCA. These results demonstrate that 2,3,4,5-tetraCA was reductively transformed by the aquifer microorganisms via the sequential removal of para and ortho halogens to yield the meta disubstituted product 3,5-diCA (Figure 2). The proposed reactions represent a major route of bioconversion since 32.5 μM 2,3,4,5-tetraCA led to the transient accumulation of 27.3 μM 2,3,5-triCA (84%) and the subsequent conversion of the latter chemical to 18.3 μM 3,5-diCA (67%). No further breakdown of the dichloroaniline isomer was observed.

Discussion

The comparison of nonsterile aquifer slurries with autoclaved controls demonstrates that the halogenated anilines were reductively transformed by biological reactions. The accumulation of the dehalogenated products occurred only in anaerobic nonsterile incubation mixtures. Furthermore, the dehalogenation of chloroanilines was only detected in the aquifer slurries obtained from the methanogenic site. This is consistent with the results of Gibson and Suflita (*38*, *39*), who demonstrated that the aryl dehalogenation of several halobenzoates, chlorophenols, and chlorinated phenoxyacetates was restricted in sulfate-reducing aquifer slurries. It is not yet clear whether the reductive dehalogenation of chloroanilines is possible under denitrifying conditions. Bollag and Russel (*40*) showed that 3,4-diCA, 3-CA, and 4-CA were suitable substrates for a *Paracoccus* sp. grown with nitrate as a terminal electron acceptor. Similarly, Mogilevich and co-workers (*41*) demonstrated the nitrate-dependent disappearance of 4-CA and 3,4-diCA by *Escherichia coli*. However, the particular pathways in these studies were not resolved.

None of the three monochloroaniline isomers were degraded in our experiments. In fact, reductive dehalogenation of chloroanilines only occurred if the parent substrates possessed at least two halogens in adjacent positions on the aromatic ring. Similar findings were reported for the reductive dehalogenation of hexachlorobenzene in sewage sludge (*42*). It is likely that the lesser halogenated anilines are more susceptible to aerobic microbial metabolism, while the polyhaloanilines are metabolized more readily under methanogenic conditions.

Several factors may help explain the preferential release of halogens from multihalogenated substrates or from certain positions relative to the aryl amino group. Brown and co-workers (*43*) calculated that the free energy released during the reductive dehalogenation of a polyhalogenated aromatic substrate is more negative (i.e., more exergonic) compared to a monohalogenated compound. For instance, the oxidation of 1 mol of glucose coupled with the reductive dehalogenation of hexachlorobenzene theoretically releases more free energy (−410.16 kcal/mol) than that of monochlorobenzene (−369.5 kcal/mol). The higher free energy associated with these substrates might preferentially favor aryl halide release from a multiply halogenated aromatic nucleus. Further, bacteria capable of benefiting from this energy release (*37*, *44*) would also likely have a selective advantage in the environment and be more easily enriched. Another factor possibly governing aryl halide removal may be the increased positive polarization of the chlorine-substituted carbon atoms when two or more adjacent halogens are positioned on the aromatic ring. Such an arrangement might destabilize the halogen–carbon bonds and render them more labile.

Lastly, the tendency of the amino group to release electrons to aromatic resonance structures makes the ring more reactive to electrophilic attack, particularly at the para and the ortho positions (*45*). This resonance effect might contribute in the preferential release of halides

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Received for review July 19, 1988. Accepted February 15, 1989. Although the research described in this article has been supported by the U.S. Environmental Protection Agency through assistance agreements No. CR-812808 and CR-813559 to the University of Oklahoma, it has not been subjected to Agency review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred.